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Purpose. Many corneal grafts undergo immune rejection, and current therapies are associated with many side effects. The purpose of this study was to identify critical chemokine pathways involved in generating the alloimmune response to corneal transplants.

Methods. Orthotopic corneal transplantation was performed in fully mismatched strains. Cytokine and chemokine receptor gene expression was determined by the RNase protection assay. Knockout (KO) strains for chemokine–chemokine receptors that are upregulated after transplantation underwent corneal transplantation. Results derived from KO murine hosts were compared with cyclosporine (Cy) therapy. In addition to graft survival, graft infiltration, allospecific delayed-type hypersensitivity (DTH), and cytokine expression were compared among the recipient groups.

Results. Initial experiments revealed gene upregulation of the chemokine receptors CCR1, -2, and -5 after corneal allorejection. Although CCR1 KO hosts showed a significant increase in graft survival compared with wild-type (WT) hosts, allografts in CCR5, CCR2/CCL3(MIP-1α), CXCR3, CXCL10/IP-10, and CCL3/MIP-1α KO mice did not show a significant improvement in graft survival. Further, CCR1 KO hosts showed a significantly higher survival rate than with systemic Cy therapy in WT hosts. Moreover, graft infiltration by leukocytes and gene expression of proinflammatory cytokines were reduced in CCR1 KO mice compared with both Cy treated and untreated WT mice, as was the induction of allospecific DTH.

Conclusions. These studies provide, for the first time, evidence that targeting of specific chemokine pathways can significantly promote survival of corneal transplants, and suggest that select deletion or suppression of CCR1 can be a useful therapeutic target in corneal transplant immunity. (*Invest Ophthalmol Vis Sci.* 2007;48:1228–1236) DOI:10.1167/iovs.05-1483

Penetrating keratoplasty has emerged as the most common form of solid tissue transplantation. Currently, more than 30,000 corneal transplantations are performed each year in the United States,[1] with a 2-year success rate as high as 90% for uncomplicated first grafts performed in avascular low-risk beds.[2,3] However, these results contrast sharply with the fate of corneal grafts placed in high-risk host beds in which rejection rates can exceed 70% to 90%, even with maximum local and systemic immune suppression.[4,5] Eyes at high risk for corneal transplant failure include those with corneal vascularization, herpes simplex keratitis, and a history of one or more previously failed grafts.[6] Although immune-mediated rejection remains the leading cause of corneal transplant failure,[6,7] surprisingly, pharmacotherapy for corneal transplantation has changed little over the past several decades, with corticosteroids remaining the mainstay of therapy. However, corticosteroids are only variably effective in either the prevention or treatment of high-risk corneal graft rejection and are capable of inducing cataracts, glaucoma, and opportunistic infections.[3,8] Development of safe and targeted new regimens that are effective in modulating alloimmune responses are therefore a priority in corneal transplantation.

The destructive immune response to corneal grafts is effected principally by delayed-type hypersensitivity (DTH)-mediating Th1 cells,[9,10] recruited into the transplant site by the local expression of chemokines.[11] Chemokines are low-molecular-weight (8–11 kDa) proteins that play a critical role in immune and inflammatory responses, with nearly 60 chemokine species and over 20 chemokine receptors identified over the past 10 years.[12] Although chemokines are best known for their ability to stimulate cell migration, they have additional activities that can contribute to tissue damage and inflammation, including enhancing T cell activation,[13] regulating Th1/Th2 polarization,[14,15] and stimulating macrophage function and protease secretion.

Work from many laboratories has demonstrated the presence of specific chemokines during the progression of allograft rejection. In the cornea, we have shown the expression of specific species, including CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CXCL10/IP-10, and CCL5/RANTES after corneal transplantation.[11] These chemokines associate with particular receptors: CCR1 with CCL3/MIP-1α and CCL5/RANTES; CCR5 with CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES; CCR2 with CCL2/MCP-1; and CXCR3 with CXCL10/IP-10. However, to date there have been no studies in which the relationship between chemokine or chemokine receptor deficiency and corneal transplant rejection has been examined. In contrast, studies in other tissues have revealed that targeted deletion of CCR1 in complete-major histocompatibility complex (MHC)–mismatched heart allografts leads to slightly increased survival compared with wild-type (WT) recipients.[16,17] Similar results have been reported for rat cardiac allografts,[18] and rabbit renal allografts.[19] In addition, targeting CCR2 has been shown to provide transient prolongation of islet cell and heart allograft

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survival.[20] When heart allografts are transplanted into CXCL10/IP-10 (a potent attractant for primed Th1 cells) knockout (KO) *recipients*, they are rejected at the same rate as their control mice. However, when heart allografts from CXCL10/IP-10 KO *donor* mice are transplanted, a significantly improved survival rate was demonstrated, which was associated with the absence of NK cell infiltration into the grafts.[21] Profound increases in survival rates were also reported in CXCR3 KO murine (the receptor for CXCL10/IP-10) recipients of heart allografts compared with WT control animals.[22] Last, targeting CCL5/RANTES, a late chemokine, through blocking antibodies to CCL5/RANTES after cardiac allografting,[23] or by using CCR5 KO recipients for cardiac allografts,[24] has also shown improvement in graft survival.

In the present study, we used as corneal transplant recipients murine KO strains of different chemokine–chemokine receptors that we had correlated with corneal transplantation. We used this method to determine more precisely which specific chemokine pathways are most robustly associated with corneal allograft rejection. We demonstrate, for the first time, that selected deletion of CCR1, but not CCR5, CCR2/CCL3(MIP-1α), CXCR3, or CXCL10/IP-10, in graft recipients, leads to significant corneal allograft survival. Further, we demonstrate that the increased survival of allografts in CCR1 KO recipients is associated with decreased expression of proinflammatory cytokines involved in allorejection, a lowered infiltration of inflammatory cells into the graft site, and suppressed DTH responses in grafted hosts.

METHODS

Animals

Unless otherwise noted, BALB/c (H-2^d) mice (Taconic Farm, Germantown, NY) were use as donors in the corneal transplantation experiments. For the chemokine receptor mRNA detection studies, C57BL/6 mice were used as recipients. Gene KO strains selected as recipients in the corneal transplantation experiments, included mice with homozygous deficiency of CCR5 (CCR5$^{-/-}$, C57BL/129 background; Jackson Laboratory, Bar Harbor, ME), CCR2/CCL3(MIP-1α) (CCR2$^{-/-}$ and CCL3/MIP-1$\alpha^{-/-}$, C57BL/129 background), CCL3/MIP-1α (CCL3/MIP-1$\alpha^{-/-}$, C57BL/6 background; Jackson Laboratory), CXCR3 (CXCR3$^{-/-}$, C57BL/6J background),[22] CXCL10/IP-10 (CXCL10/IP-10$^{-/-}$, C57BL/129 background), and CCR1 (CCR1$^{-/-}$, C57BL/129 background).[25] The wild-type control mice (C57BL/129 and C57BL/6) were procured originally from the Jackson Laboratory and subsequently bred at the Schepens Eye Research Institute animal colony. In most experiments, male mice were used that were 6 to 12 weeks of age. Each animal was placed under general anesthesia by intramuscular injections of ketamine (3–4 mg) and xylazine (0.1 mg) for each surgical procedure. All protocols were approved by the Institutional Animal Care and Use Committee. Animals were housed under specific pathogen-free conditions and were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Transplantation

Our standard protocol for murine orthotopic corneal transplantation was used for these studies.[26] Briefly, the center of the donor cornea (from wild-type BALB/c background mice) was marked with a 2-mm diameter microcurette, excised with Vannas scissors (Storz Instruments Co., St. Louis, MO) and placed into chilled phosphate-buffered saline (PBS). The recipient graft bed (chemokine or chemokine receptor KO mice, C57BL/129 WT or C57BL/6 WT mice) was prepared by excising a 1.5-mm site in the central cornea. The donor button was then placed onto the corneal bed of recipients and secured with eight interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). Antibiotic ointment was applied, followed by a 24-hour tarsorrhaphy with 8-0 nylon sutures (Sharpoint). Normal (ungrafted) C57BL/129 or C57BL/6 hosts were used as control mice. Graft sutures were removed in all cases on day 7. Grafts were evaluated biomicroscopically in a masked fashion for at least 8 weeks every 2 to 3 days, and graft opacity and neovascularization were measured according to a standardized scheme in which grafts with an opacity score of 2+ or greater after 3 weeks were considered rejected.[9] Transplants with an opacity score of 3+ or greater after 2 weeks that never cleared by 8 weeks were also regarded as rejected. Mice with surgical complications (cataract, significant anterior synechiae or failure to form an anterior chamber by day 2) were excluded from the study. Immunosuppression with cyclosporine (Cy; a kind gift from Mohamad H. Sayegh, Harvard Medical School, Boston, MA) was afforded by dissolving Cy in olive oil and administering it daily (10 mg/kg intraperitoneally) for 14 days, beginning at the time of transplantation. Both isografts and normal (ungrafted) host mice were used as control animals in some experiments.

RNA Preparation and RNase Protection Assay of Chemokine Receptor Expression and Cytokine Expression

At select time points after corneal transplantation (n = 6–10 corneas per experiment for cytokine template, and n = 4–5 whole eyes for chemokine receptor template), total RNA was extracted by the single-step method using RNA-STAT-60 (Tel-Test Inc., Friendswood, TX). Briefly, whole corneas or whole eyes were homogenized and centrifuged to remove cellular debris. The RNA pellet was resuspended in nuclease-free water and processed together as a group. Detection and quantification of murine chemokine receptor mRNAs were performed with a multiprobe RNase protection assay (RPA) system (BD Bioscience, Franklin Lake, NJ), as recommended by the supplier and as we have previously described.[11] Briefly, a mixture of [α-^{32}P] UTP-labeled antisense riboprobe was generated from the chemokine receptor template set mCR-5 (3 weeks after transplantation) and the cytokine template set mCK1b (2 weeks after transplantation; to analyze the cytokine profile prerejection; BD Bioscience). Ten micrograms of total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the ^{32}P-anti-sense riboprobe mixture. After purification by ethanol precipitation, the samples were resolved on 5% polyacrylamide sequencing gels. The gels were dried and subjected to autoradiography. Protected bands were observed after exposure of gels to x-ray film. The bands were quantitated by densitometric analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Both isografts and normal (non-grafted) host mice were used as control animals. All samples were analyzed in duplicate.

Subconjunctival Injection of Anti-RANTES Blocking Antibody

Corneal transplantation (n = 33) was performed from C57BL/6 mice (donor) to BALB/c mice (recipient) as just detailed. After routine general anesthesia, subconjunctival injection was performed with a 100-μL glass syringe (Hamilton Co., Reno, NV) and a 30-gauge needle (n =10). A total of 5 μL (5 μg each) of rat anti-mouse CCL5/RANTES blocking Ab (clone 53405; R&D Systems, Minneapolis, MN) was injected on the day of surgery, and at 4, 7, 10, 14, 21, and 25 days after surgery. The same amount of rat IgG2$_a$ (clone 54447; R&D Systems) was used as a control (n =12). The remaining mice (n =11) did not receive any treatment.

Histopathological Evaluation of Graft Infiltration

For the measurement of leukocytic infiltration of grafts before any clinical signs of rejection, wild-type and KO mice were killed at days 7 and 14 (each, n =5) after transplantation and the grafted eyes were enucleated. The enucleated eyes were fixed with 10% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. At least 10 sections per eye were evaluated morphologically by light microscopy in a masked fashion, and the total number of

leukocytes, polymorphonuclear cells, and mononuclear cells was counted per section.

Antibodies and Immunohistochemistry

The immunohistochemical staining procedures were performed using the following Abs: purified hamster anti-mouse CD11c (HL3, dendritic cell marker); FITC-conjugated hamster anti-mouse CD3-e (145-2C11; T-lymphocyte marker); purified rat anti-mouse CD45 (30-F11, panleukocyte marker); purified goat anti-mouse CCR1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and purified goat anti-mouse CCR5 (M-20; Santa Cruz Biotechnology). The secondary antibodies were rhodamine-conjugated goat anti-rat IgG, FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), and Cy5-conjugated goat anti-Armenian hamster IgG. Isotype controls included rat IgG_{2b}, and hamster IgG. All primary and secondary mAbs (except where noted) and isotype-matched controls were purchased from BD PharMingen (San Diego, CA).

For the histochemical studies, normal and grafted corneas were excised from C57BL/129 or CCR1 KO mice, and the epithelium was removed from the stroma as described before.[27] Full-thickness corneal stromal tissue (for CCR1, CCR5, CD11c, and CD45), corneal epithelial sheets[28] (for CCR1, CCR5, CD11c, and CD45), or 8-μm frozen sections (for CD3) were fixed in acetone for immunofluorescence staining as previously described.[29] In brief, sections, flatmounts, or epithelial sheets were immunostained with primary antibodies or isotype-matched control antibodies for 2 hours. Thereafter, the samples were incubated with secondary antibodies for 1 hour if necessary. All staining procedures were performed at room temperature, and each step was followed by three thorough washings in PBS for 5 minutes each. The samples were covered with mounting medium (Vector Laboratories, Burlingame, CA) and analyzed by confocal laser scanning microscope (Leica TCS 4D; Lasertechnik, Heidelberg, Germany). At least five sections ($n = 3$ animals) or three corneal flatmounts were analyzed for each experiment.

Assessment of Donor-Specific DTH

At 3 weeks after corneal transplantation, 1×10^6 irradiated (2000 rad) spleen cells from donors syngeneic with the corneal grafts (BALB/c), in 10 μL Hanks' balanced salt solution, were injected into the right pinnae of corneal hosts.[30] As a control, a similar number of spleen cells were injected into the ear pinnae of wild-type and CCR1 KO mice that had been immunized 1 week earlier by intraperitoneal injection of 10×10^6 spleen cells of the appropriate allogeneic strain. All experimental and control groups consisted of five animals. After 24 and 48 hours, ear thickness was measured in a masked fashion with a low-pressure micrometer (Mitutoyo; MTI Corp., Paramus, NJ). Allospecific ear swelling was expressed as follows: specific ear swelling = (24-hour measurement of right ear − 0 hour measurement of right ear) − (24-hour measurement of left ear − 0 hour measurement of left ear) × 10^{-3} mm. Ear-swelling responses are presented as mean ± SE. Because results at 24 and 48 hours were similar, only 24-hour data are presented. Both isografts as well as normal (non–graft recipient) host mice were used as control subjects.

Statistical Analyses

Rates of corneal graft survival were plotted as Kaplan-Meier survival curves and were compared using the log-rank (Mantel-Cox) or Student's *t*-test for specific time point for KO mice or Cy-treated mice compared with the WT control samples. mRNA expression levels are given as the mean ± SE. Student's *t*-test was used for comparison of DTH responses and graft infiltration. Statistical significance was defined as $P < 0.05$.

Results

Chemokine Receptor Gene Expression in High-Rejecting C57BL/6 and Low-Rejecting BALB/c Hosts

Orthotopic corneal transplantation was performed in two recipient wild-type murine models characterized by a low (C57BL/6 to BALB/c; 50% rejection; median time to rejection, 28 days) or high (BALB/c to C57BL/6; 90% rejection; median time to rejection, 21 days) rate.[31] Naïve mice and isografts were used as control subjects. Whole eyes were excised 3 weeks after transplantation, and RPA was performed to detect increased chemokine receptor gene expression. Figures 1A–C demonstrate the results of the RPA autoradiograph and the quantity of chemokine receptor mRNA normalized to GAPDH. There was an increased expression of CCR1, -2, and -5 mRNA in rejected allografts, both in the C57BL/6 (Fig. 1B) and BALB/c hosts (Fig 1C). CCR1 and CCR2 mRNA were also minimally detected in accepted allografts in both models (Figs. 1B, 1C). Isografts and naïve mice demonstrated no increase in chemokine receptor expression.

Corneal Graft Survival in Chemokine and Chemokine Receptor KO Mice

Given the overexpression of CCR1, -2, and -5 genes, and the previously demonstrated gene overexpression of several of their ligands and the CXCL10/IP-10 gene (a ligand for CXCR3) in rejected allografts,[11] we tested the effects of genetic deletion of CCR1, CCR2, CCR5, CXCR3, CCL3/MIP-1α, and CXCL10/IP-10 on corneal graft survival in KO mice, using fully mismatched strains. The results are summarized in Table 1. Corneal allografts in CCR5 KO mice showed no improved survival at 8 weeks (11%) compared with WT mice (10%; $P = 0.94$). There was, however, a slight delay in rejection as evidenced by the survival rate at 4 weeks after transplantation (44% in CCR5 KO mice versus 20% in WT mice; $P = 0.28$). Graft survival in CCR2/CCL3(MIP-1α) double-KO, CCL3/MIP-1α KO, CXCL10/IP-10 KO, and CXCR3 KO mice did not show any improvement compared with graft survival in the respective WT mice at any time point studied (Table 1). However, corneal grafts in CCR1 KO mice showed a significantly improved threefold graft survival compared with WT hosts. The survival rate in CCR1 KO hosts (C57BL/6 background) was 100% at 4 weeks and 60% at 8 weeks compared with a survival rate of 30% and 20% in WT mice, respectively ($P = 0.001$ and $P = 0.004$ respectively).

Corneal Allograft Survival in CCR1 KO Hosts Compared with Systemic Cy Treatment

To evaluate the corneal graft survival in CCR1 KO mice compared with conventional therapy, we treated wild-type hosts with a 14-day course of Cy at 10 mg/kg per day intraperitoneally[17] and compared the graft survival rate in these mice to CCR1KO hosts (Fig. 2). Corneal transplants were followed daily for 10 weeks. Corneal graft survival in CCR1 KO hosts was significantly higher at 10 weeks (60%) compared with WT mice treated with Cy (30%; $P = 0.01$) and untreated mice (20%; $P = 0.002$).

Role of CCL5/RANTES in Corneal Allograft Rejection

To determine the role of CCL5/RANTES, a ligand to both CCR1 and -5 receptors, in corneal allograft rejection, blocking experiments were performed after corneal transplantation with sub-

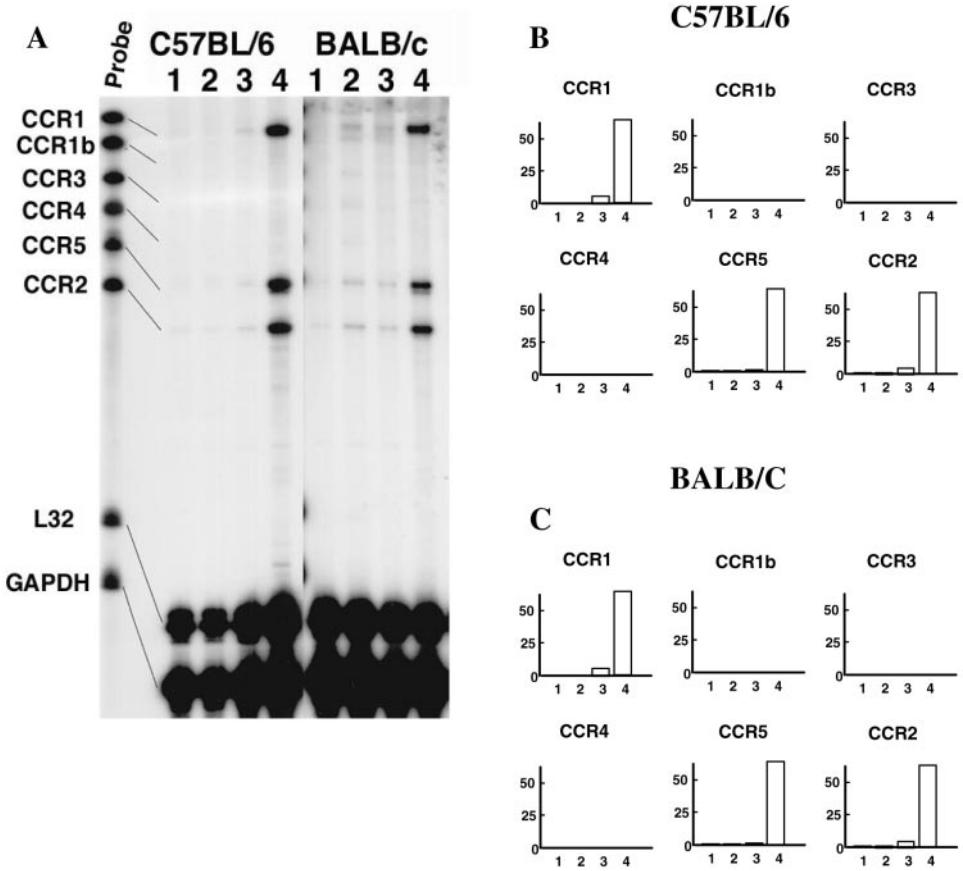

FIGURE 1. CC chemokine receptor gene expression levels 3 weeks after corneal transplantation. Autoradiography data for C57BL/6 and BALB/c hosts are shown in (**A**). On the basis of the migration pattern of the undigested probes, specific bands were identified for each chemokine. Densitometric analyses normalized to GAPDH are shown in (**B**) for C57BL/6 hosts and in (**C**) for BALB/c hosts. Vertical axes indicate arbitrary units based on densitometry. Overexpression of CCR1, -2, and -5 mRNA was observed 3 weeks after corneal transplantation in both models. Two sets of results on separate experiments were analyzed, and averaged density was shown as the results of densitometric analysis. 1, naïve mice; 2, isografts; 3, accepted allografts; 4, rejected allografts.

conjunctival injection of RANTES neutralizing Ab. Subconjunctival injections were performed on days 0, 4, 7, 10, 14, 21, and 25. There was no improvement in corneal graft survival in mice treated with a CCL5/RANTES-blocking Ab (n =10; 6/10 [60.0%] rejected) compared with mice injected with isotype control $IgG2_a$ (n =12; 7/12 [58.3%] rejected) or untreated control animals (n =11; 5/10 [50.0%] rejected).

Profile of Corneal Leukocyte Infiltration

To elucidate the potential mechanisms by which the deletion of CCR1 influences corneal graft outcome, graft infiltration profiles were compared in grafts performed in CCR1 KO mice and in Cy-treated and untreated WT mice. Corneas were excised at 7 and 14 days after transplantation, and sections were

TABLE 1. Survival of Fully Mismatched Corneal Allografts in Different Strains of Chemokine/Chemokine Receptor KO Recipients and Controls (percent survival)

	Weeks after Surgery		
Strain (Animals)	**2**	**4**	**8**
CCR5-KO	9/9 (100)	4/9 (44)	1/9 (11)
CCR5-WT	9/10 (90)	2/10 (20)	1/10 (10)
P	0.36	0.28	0.94
CCR2/CCL3(MIP-1α)-KO	11/11 (100)	1/11 (9)	0/11 (0)
CCR2/CCL3(MIP-1α)-WT	9/10 (90)	2/10 (20)	1/10 (10)
P	0.31	0.5	0.31
CCL3/MIP-1α-KO	6/8 (75)	0/8 (0)	0/8 (0)
CCL3/MIP-1α-WT	6/8 (75)	0/8 (0)	0/8 (0)
P	1	1	1
CXCL10/IP-10-KO	6/10 (60)	0/10 (0)	0/10 (0)
CXCL10/IP-10-WT	8/10 (80)	0/10 (0)	0/10 (0)
P	0.17	1	1
CXCR3-KO	7/7 (100)	7/7 (100)	2/7 (29)
CXCR3-WT	7/7 (100)	6/7 (86)	3/7 (43)
P	1	0.36	0.36
CCR1-KO	10/10 (100)	10/10 (100)	6/10 (60)
CCR1-WT	7/10 (70)	3/10 (30)	2/10 (20)
P	0.08	0.001	0.037

* CCR1-KO data are plotted in Figure 2.

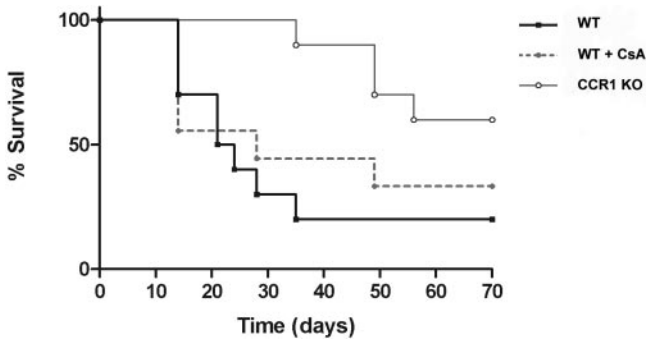

FIGURE 2. Kaplan-Meier graft survival curves for allogeneic corneal transplants. Grafts are shown for CCR1 knockout hosts (*blue*), wild-type mice with cyclosporin treatment (*red*), and wild-type control mice (*black*). There was a significant delay in rejection in CCR1 KO hosts. Moreover, there was a significantly higher survival rate in CCR1 KO mice compared with cyclosporin-treated and control mice.

subjected to hematoxylin and eosin (H&E) staining for morphologic enumeration of mononuclear and polymorphonuclear (PMN) cells (Fig. 3). Leukocyte (PMN and mononuclear) infiltration decreased in all three models between days 7 and 14. However, there was a profound (>50%) decrease in the number of leukocytes infiltrating transplants placed in CCR1 KO mice compared with WT mice and Cy-treated mice (Fig. 3A; $P < 0.05$) and this was evident for both PMN (Fig. 3B; $P < 0.001$) and mononuclear (Fig. 3C; $P < 0.05$) cells. Further, the difference in mononuclear cell infiltration was significant ($P < 0.05$) between the two groups at both time points studied (Fig. 3C). Results for rejected WT mice are given at day 14 only for comparison. Although macrophages are known to constitute the prominent early component of graft infiltration, we also evaluated the effect of CCR1 gene deletion on the number of infiltrating T cells, based on the membrane expression of CD3 on days 6 and 12 after transplantation, before overt clinical graft rejection, which started at approximately around day 14 (Fig. 4). At day 6 after transplantation, $CD3^+$ cells were nearly absent in all groups (Fig. 4A) with no significant difference between the WT mice compared with CCR1 KO mice ($P = 0.058$) or WT mice compared with WT mice treated with Cy ($P = 0.11$). By day 12, there was a significant influx of $CD3^+$ cells into the WT hosts (Fig. 4B), whereas CCR1 KO ($P = 0.014$; compared with WT mice) and Cy-treated ($P = 0.003$; compared with WT mice) groups demonstrated no such influx. The difference between CCR1 KO mice compared with Cy-treated WT mice was not significant at both 6 ($P = 0.051$) and 12 ($P = 0.133$) days after transplantation.

CCR1 Expression on Graft Cells

Evaluation of infiltrating leukocytes demonstrated that most of these cells constitute mononuclear cells that include macrophages and dendritic cells (DCs). Normal corneas were excised form WT mice and stromal wholemounts, as well as epithelial sheets, were double stained for CD45 (pan-leukocyte marker) or CD11c (DC marker) and CCR1 or CCR5, and evaluated by confocal microscopy. Results demonstrated the absence of CCR1 and CCR5 expression by stromal DCs and epithelial LCs in normal ungrafted corneas (data not shown). Next, corneal transplantation was performed in WT mice, and the corneas were excised at 3 and 6 days after transplantation. Corneal stromal wholemounts and epithelial sheets were double stained for CD45 or CD11c and CCR1 or CCR5 (Fig. 5). Although at 3 days no staining was detected for CCR1 or CCR5 in either epithelium or stroma, CCR 5 expression was detected at day 6 after transplantation in the epithelium (Figs. 5A–C), similar to CCR5 expression on epithelial LCs as previously demonstrated.[32] CCR1 expression was not detected at any time point studied on epithelial LCs (Figs. 5D–F). An increase in CCR5 (Figs. 5G–I) and CCR1 (Figs. 5J–L) expression was detected at days 3 and 6 after transplantation on stromal $CD11c^+$ DCs (Figs. 5G–L).

Suppression of Th1 Cytokines and DTH Responses in CCR1 KO Recipients

To assess the extent to which deletion of CCR1 gene expression affects production of Th1 cytokines before rejection of allografts at 3 weeks after transplantation, corneas were excised 2 weeks after transplantation (before the onset of allorejection) and examined for Th1 cytokine gene expression

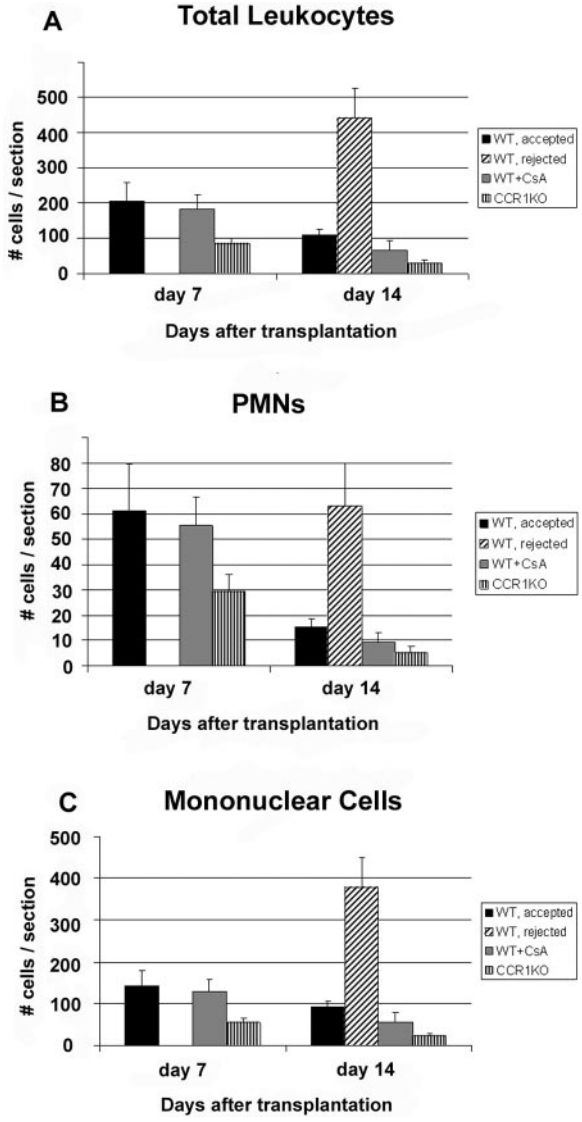

FIGURE 3. Leukocyte infiltration into corneal allografts. Allogeneic (BALB/c) corneal grafts were performed in CCR1 KO ($n = 10$), untreated WT ($n = 10$), and Cy-treated WT mice ($n = 10$). At days 7 ($n = 5$ per group) and 14 ($n = 5$ per group), corneas were harvested and the leukocytic infiltration quantified (**A**) and subtyped morphologically with light microscopy for polymorphonuclear cells (PMN) (**B**) and mononuclear cells (**C**). Data depicted as the mean ± SE. Significantly decreased infiltration of total leukocytes, PMN and mononuclear cells was observed into CCR1 KO hosts compared with Cy-treated and untreated wild-type hosts at both time points studied.

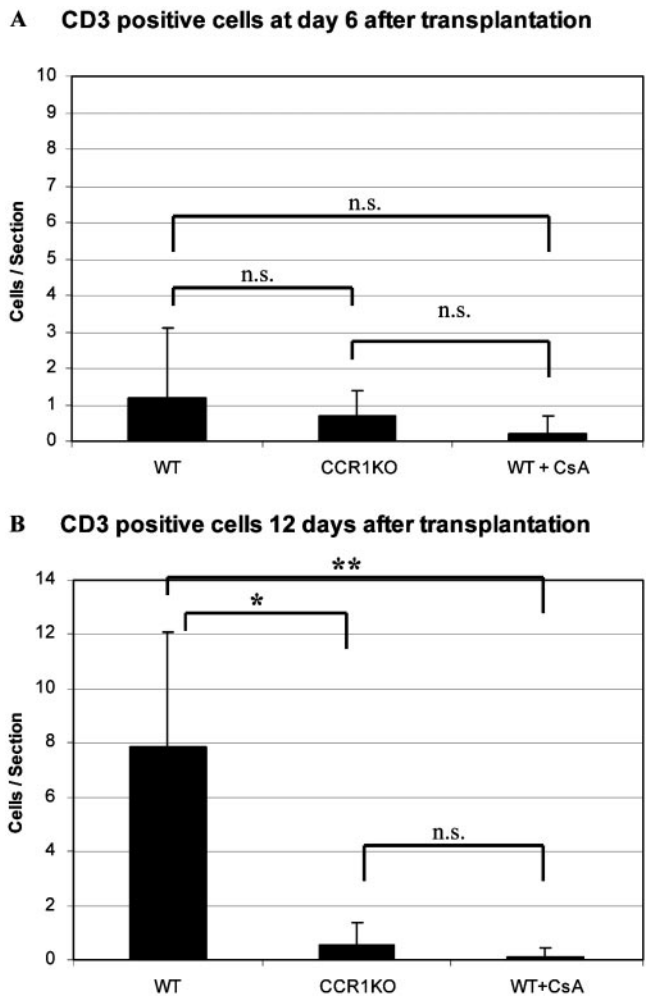

FIGURE 4. T-cell infiltration into corneal allografts. Allogeneic (BALB/c) corneal grafts were performed in CCR1 KO, cyclosporin-treated, and untreated WT mice. At days 6 (**A**) and 12 (**B**), corneas were harvested and T-cell infiltration quantified based on immunohistochemical staining with anti-CD3. At day 6 after transplantation (**A**), $CD3^+$ cells were nearly absent in all groups. By day 12, there was significant influx of $CD3^+$ cells into WT hosts (**B**), whereas CCR1 KO and cyclosporin-treated groups demonstrated no such influx. Data depicted as the mean ± SE; $^*P = 0.014$, $^{**}P = 0.003$, NS = $P > 0.05$.

by RPA, in both CCR1 KO and WT recipients. Results demonstrated a suppression of Th1 cytokines interleukin (IL)-2 and interferon (IFN)-γ in CCR1 KO mice compared with WT hosts (Fig. 6). Although the level of IL-2 expression in CCR1 KO recipients 2 weeks after transplantation was negligible and comparable to that in naïve mice, the level of IFN-γ was reduced by half in CCR1 KO recipients compared with WT mice (Fig. 6).

To examine whether CCR1 KO transplant recipients are sensitized against donors, we performed allospecific DTH studies among CCR1KO and WT recipients (Fig. 7). Naïve mice served as negative controls, and mice that were previously sensitized to donor cells served as positive controls. The DTH studies were performed at 3 weeks after transplantation, when graft recipients are known to have a positive DTH response. Compared with WT hosts (mean, 93 μm), CCR1 KO hosts (mean, 52 μm) demonstrated a significant ($P < 0.05$) decrease in DTH alloreactivity, indicating suppressed sensitization in CCR1 KO mice (Fig. 7).

DISCUSSION

Corneal transplantation has restored sight to millions of blind people worldwide, but there have been no major changes in pharmacotherapy for decades since the advent of corticosteroids and most high-risk grafts are still rejected despite maximum anti-inflammatory therapy. Deletion or blockade of molecular mediators of alloimmunity potentially offers the possibility of more precise inhibition with fewer side effects. The deletion or blockade of chemokines has been partially successful in several organ transplantations,[16–24,33] but mechanistic studies unraveling chemokine pathways in corneal transplantation have not been performed thus far. The present study demonstrates, for the first time, that targeting the chemokine receptor CCR1 can lead to a significant increase in long-term corneal graft survival.

Our previous work evaluating patterns of chemokine expression in the late phase of corneal transplant rejection identified CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CCL2/MCP-1, and CXCL10/IP-10 mRNA overexpression, though the functional relevance of these findings in the immunopathogenesis of graft rejection was not investigated.[11] In this study, we focused on the expression and functional role of specific CC and CXC chemokine receptors implicated in corneal alloimmunity by virtue of their overexpression and examined the effect of selective gene deletion to demonstrate that CCR1 suppression had a significant effect in promoting allograft survival, even beyond that seen with Cy administration.

In light of our previous findings, the current findings may be surprising, since the deletion of CCR1 led to a significantly improved allograft survival, whereas deletion of other chemokine receptors, whose ligands were upregulated after graft rejection did not. Although the results obtained by Yamagami et al.[11] were obtained from whole eyes that included both donor and recipient tissue, the current experiments were performed using recipient KO mice. Hancock et al.[21] showed elegantly that when CXCL10/IP-10 KO allografts were transplanted into WT recipients, a significantly improved survival rate was demonstrated. In contrast, the survival was not improved when WT allografts were transplanted into CXCL10/IP-10 KO recipients. Therefore, the KO mice used herein as recipients might demonstrate improved corneal graft survival if used as donors. In addition, there is a high level of promiscuity in the chemokine system. Despite this promiscuity, there are functional differences when the same ligands bind to different receptors. This is, in part, because different chemokine receptors are expressed by different cell types. We show here that in addition to CCR1 KO mice, the CCR5 KO mice recipients (CCR1 and CCR5 share ligands) demonstrated a delayed rejection compared with WT mice. The role of CCR5 and CCL5/RANTES in recruitment of MHC class II-positive LCs in the corneal *epithelium* has been shown by us recently.[30] Our current data suggest that while some stromal DCs also express CCR5, they are the only antigen-presenting cell (APC) population that express CCR1, because epithelial LCs do not express this receptor. This finding underscores the importance of intrastromal graft infiltration by APCs in corneal alloimmunity. Moreover, the different expression patterns of CCR1 and CCR5 by the epithelial and stromal DCs could explain the different results obtained between CCR1 KO and CCR5 KO mice in this study. Similarly, previous studies have shown that chemotactic migration of monocytic-derived DCs (like corneal stromal DCs), is abolished with Abs to CCR1, but not with Abs to CCR5.[34] The different roles of epithelial LCs and stromal DCs are further confirmed by the fact that, although CCL5/RANTES blockade led to a CCR5-mediated suppression in LC migration after inflammation, CCL5/RANTES blockade alone did not im-

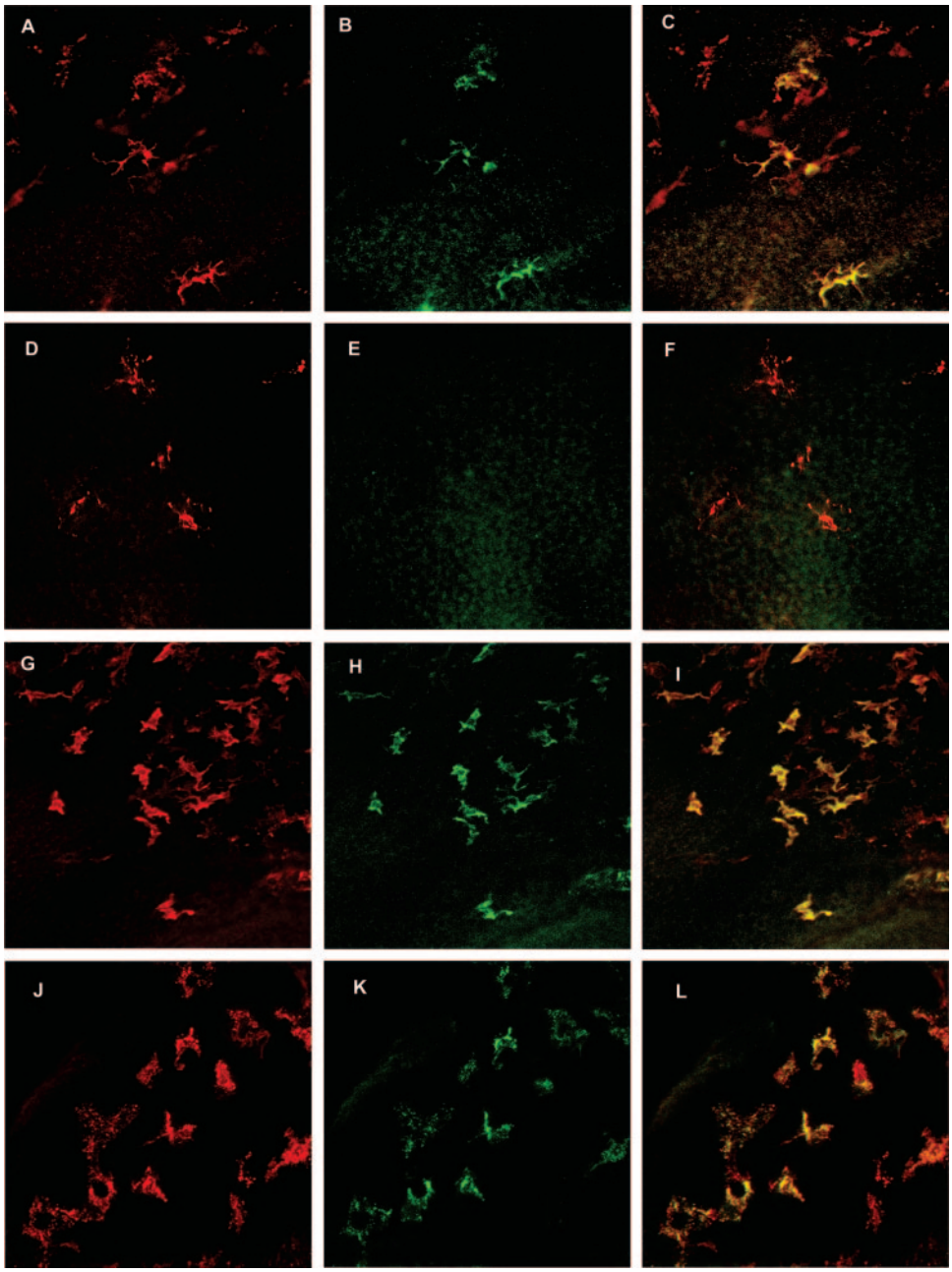

FIGURE 5. Immunohistochemical studies of epithelial sheets and stroma by confocal microscopy for CCR1 and -5. On day 6 after corneal transplantation, $CD11c^+$ LCs (*red*) (**A**, **D**) expressed CCR5 (*green*) (**B**), but not CCR1 (*green*) (**E**). Dendritic cells (*red*) (**G**, **J**) expressed CCR5 (*green*) (**H**) and CCR1 (*green*) (**K**) in the corneal stroma. (**A**, **D**, **G**, **J**) $CD11c^+$ (*red*), (**B**, **H**) $CCR5^+$ (*green*), (**E**, **K**) $CCR1^+$ (*green*), (**C**, **F**, **I**, **L**) merged, costained (*yellow*). (**A**–**F**) Stained epithelial sheets; (**G**–**L**) stained stromal wholemounts.

prove corneal graft survival, suggesting that other chemokines are involved in the CCR1-mediated corneal allograft rejection.

Corneal allograft rejection is primarily mediated by $CD4^+$ Th1 cells.[35–37] This study demonstrates the mediation of Th1 responses by CCR1, as CCR1 deletion was associated with a reduced influx of T cells into the cornea, as well as abolished gene expression of Th1 cytokines IL-2 and IFN-γ. IFN-γ, a product primarily of Th1 cells, can induce production of Th1-attracting chemokines, whereas antagonizing Th2-attracting chemokines.[38] CXCL10/IP-10 for instance, is induced by IFN-γ and is expressed abundantly in rejected corneal transplants.[11] IFN-γ can also synergize with IL-1 and TNF-α to stimulate chemokine production, and as such, demonstration of the critical role of CCR1 in mediating corneal allograft rejection provides indirect confirmation for our previous work implicating IL-1 and TNF-α in alloimmunity.[30,32,35,39,40]

Leukocyte recruitment into the corneal graft is a major component of the early alloimmune response, as is the case for other tissue grafts. A recent study demonstrated in a renal inflammatory model that CCR1, but not CCR5, is required for leukocyte recruitment.[41] Further, Eis et al. demonstrated through the use of the CCR1- antagonist BX471 and CCR5-blocking antibodies that CCL5/RANTES-induced arrest of monocytes and T cells was predominantly mediated by CCR1 but not CCR5.[41] In addition, neutrophils have been reported to express CCR1, explaining our observation that CCR1 blockade has a profound effect on neutrophil infiltration into corneal grafts. Earlier studies have shown that neutrophils from CCR1 KO mice are nonresponsive to CCL3/MIP-1α, suggesting that CCR1 may be the dominant CCL3/MIP-1α binding receptor subtype in these cells.

The process of antigen presentation is an indispensable step in the corneal alloimmune response, and our knowledge regarding the presence and importance of corneal APCs has evolved over the past years.[42,43] Here, we confirm our previous findings that CCR5, but not CCR1, is expressed on

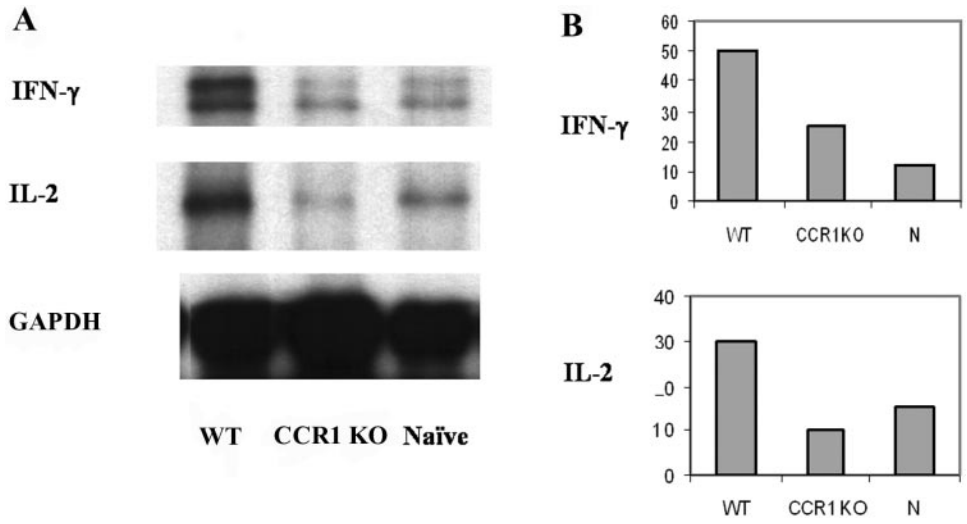

FIGURE 6. Th1 cytokines are decreased in CCR1 KO hosts. Corneas of CCR1 KO and WT hosts were excised 2 weeks after transplantation, and RPA was performed to detect gene expression levels of Th1 cytokines in the transplanted corneas before rejection. (**A**) Autoradiograph data. On the basis of the migration pattern of the undigested probes, specific bands were identified for each cytokine. Densitometric analyses normalized to GAPDH are shown in (**B**) for CCR1 KO hosts and in WT hosts. Results demonstrate suppression of both IL-2 and IFN-γ in CCR1 KO mice compared with WT mice.

$CD11c^+CD11b^-$ *epithelial* LCs during inflammation.[32] Further, we noted that *stromal* DCs (that are uniformly $CD11b^+$) are able to express both CCR1 and -5 during inflammation, and hence are the only APCs that express CCR1 in the cornea. CCR1 and -5 are responsible for the recruitment of immature DCs in a variety of inflamed tissues, through the chemokines CCL3/MIP-1α, CCL5/RANTES, and CCL7/MCP-3. Once activated, DCs secrete high levels of CCL3/MIP-1α (not made constitutively by resident corneal cells)[11,44] that lead to additional recruitment of inflammatory cells. This is a likely explanation for the significant effect of CCR1 (but not CCR5) blockade on allograft infiltration and rejection, indicating the important role of stromal DCs in the alloimmune response. In addition, given the critical role of APCs in initiating the alloimmune response, the decreased recruitment of DCs observed in the absence of CCR1 inhibits the initiation and sustained Ag sampling, thereby decreasing or delaying the priming of effector T cells. The role of CCR1, but not CCR5, in regulating the interaction of monocytic-derived DCs and T cells has been demonstrated in the past.[34]

It is important to address the potential limitations in this study. First, we primarily extracted RNA from whole-eye homogenates for analysis of chemokine receptor mRNA to prevent the problems faced with the very small quantities of RNA extracted from the murine cornea. The use of only corneal RNA would have translated into a significant increase in the number of animals used. We had faced similar problems in our previous studies where we confirmed that the expression of specific chemokine mRNA after allograft rejection that was reflected in the whole-eye data was similar to data in the corneal microenvironment.[11] Therefore, although the RPA analysis of whole eyes has the disadvantage of not limiting the assay to the cornea alone, it has the distinct advantage of also assaying chemokine species expressed by other (noncorneal) structures in the anterior segment, such as the vascularized tissues in and around the iris root that also materially contribute to leukocyte infiltration into the anterior eye structures, including the cornea and anterior chamber after corneal transplantation.

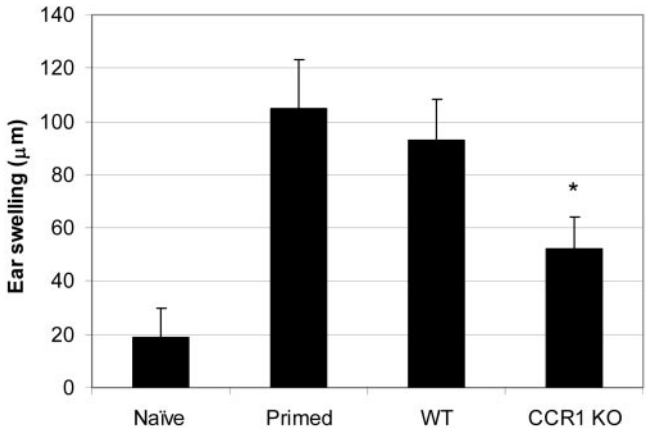

FIGURE 7. Donor-specific DTH in CCR1 KO hosts is decreased. Ear swelling of naïve (negative control), subcutaneous primed (positive control) WT hosts, and CCR1 KO hosts were measured with a micrometer 24 and 48 hours after ear challenge by donor cells 3 weeks after transplantation. CCR1 KO hosts demonstrated a significantly suppressed degree of donor-specific ear swelling (DTH) compared with control hosts ($^*P < 0.05$).

Second, although the deletion of CCR1 clearly modulated corneal graft rejection in CCR1 gene-targeted recipient mice, some mice still rejected their corneal grafts. Although leukocyte recruitment into a corneal graft is a major component of the alloimmune response, corneal allograft rejection is controlled by $CD4^+$ T cells. We demonstrated here that corneal allograft survival can be prolonged through a substantial decrease in the number of infiltrating T cells in CCR1 KO recipients compared with WT recipients. However, the fact that some CCR1 KO recipients still rejected their allografts, demonstrates that CCR1-mediated mechanisms, despite their significance, alone cannot entirely account for the alloimmune response. Finally, although we used CCR1 KO recipients in this study to analyze the role of CCR1, we have not used an inhibitor/antagonist of CCR1 in WT recipients to confirm these results, and therefore we cannot comment on alternate (e.g., compensatory) pathways that may exist in the CCR1 KO mice. However, notwithstanding this limitation, it is important to emphasize that these compensatory pathways are typically more problematic in a setting of "false-negative" data with KO animals, in which they can compensate for the lack of a gene through changed expression of other gene products, rather than in this case in which specific gene deletion led to a significant change in outcome.

In summary, this study is the first to identify the role of a critical chemokine receptor in corneal alloimmunity. Targeting CCR1 alone or its ligands may prove to be an effective strategy to suppress the rejection of corneal transplants, thus reducing

the risk of the myriad toxic side effects of alternative nonspecific immunosuppressive drugs.

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